DECLARATION

I, Mariko MATSUKAWA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Bldg., 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on February 19, 2004 under Patent Application No. 043481/2004 and that the following is a true and correct translation to the best of my knowledge and belief.

Dated: January 23, 2009

M. Matsukawa

Mariko MATSUKAWA

[Document's Name] Application for Patent

[Reference Number] A41108J

[Filing Date] February 19, 2004

[Address] To the Commissioner of the Patent Office

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[Representative] Masazumi IMAMURA

[Fee]
[Number of register of payment] 170347
[Amount] 21,000

[List of Attached Documents]

[Document's Name]	Claims 1
[Document's Name]	Specification 1
[Document's Name]	Drawings 1
[Document's Name]	Abstract 1
[Number of Comprehensive Power of Attorney]	0209669

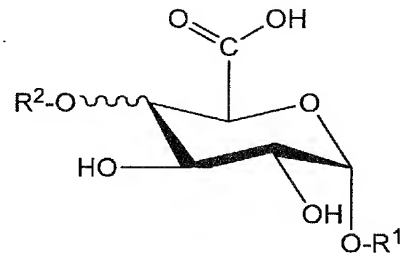
[Document] Claims

[Claim 1]

A composition for NKT cell activation comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

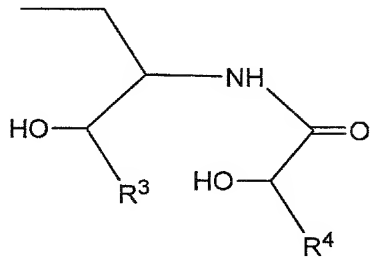
[Formula 1]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 2]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

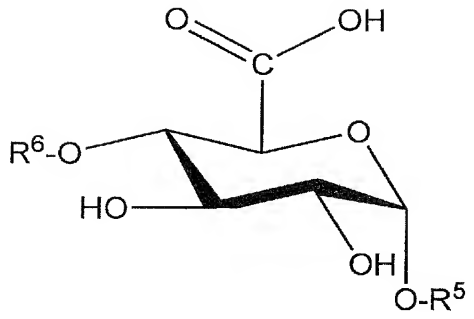
R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.

[Claim 2]

A composition for NKT cell activation comprising a glycosphingolipid having a structure represented by the following formula (3):

formula (3)

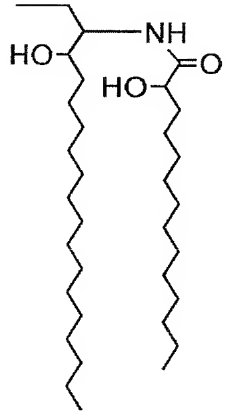
[Formula 3]



wherein R^5 represents R^{51}, R^{52}, or R^{53}; and R^6 represents hydrogen, R^{62}, R^{63}, R^{64}, or R^{65}:

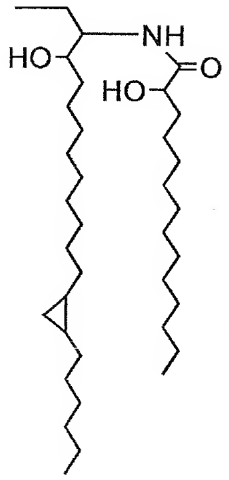
R^{51}:

[Formula 4]



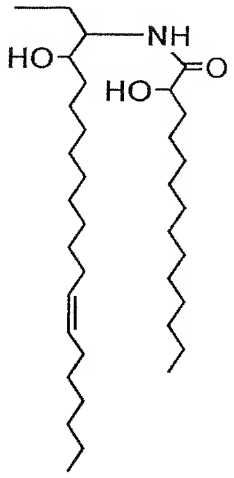
R^{52}:

[Formula 5]



R^{53}:

[Formula 6]



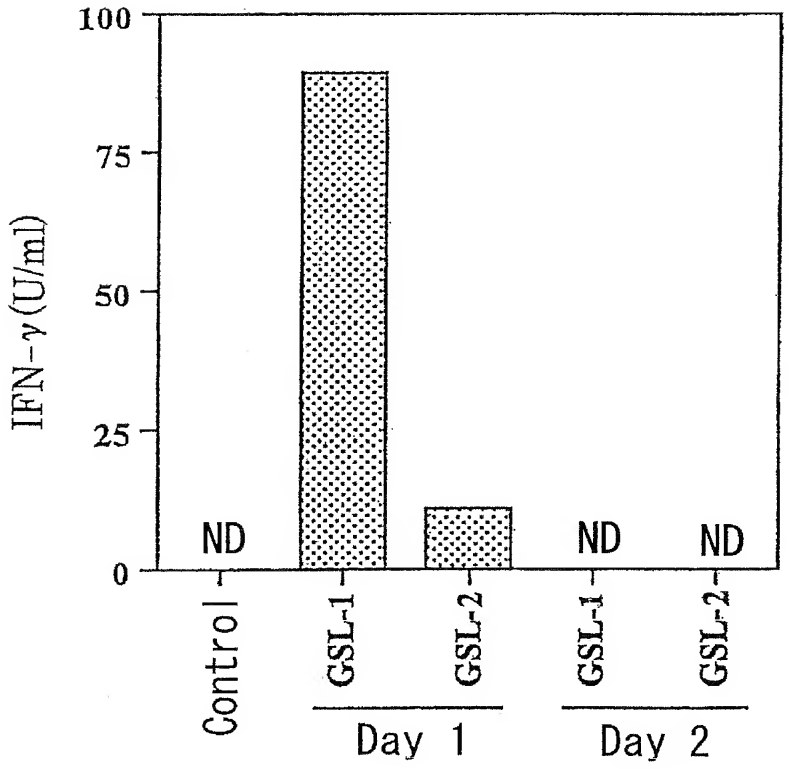
R^{62}:

[Formula 7]

	Day 1 (%)	Day 2 (%)
Control	14.5	13.7
GSL-1	18.4	22.8 *
GSL-2	21.7 *	26.1 *
GSL-6	14.6	23.4 *
GSL-7	11.9	23.2 *

R^{63}:

[Formula 8]



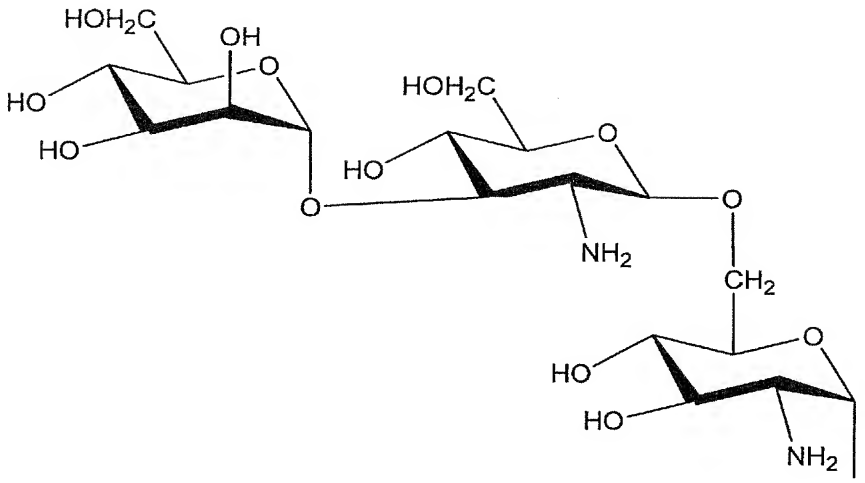
R^{64}:

[Formula 9]

	Day 1(%)	Day 2(%)
Control	3.4	3.4
GSL-1	9.8	35.5
GSL-2	24.0	25.9

R^{65}:

[Formula 10]

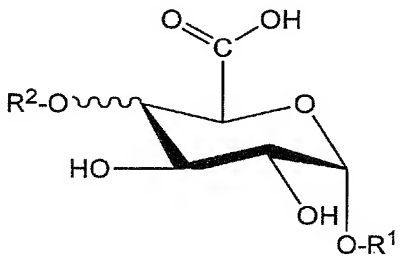


[Claim 3]

A composition for accelerating IL-4 production comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

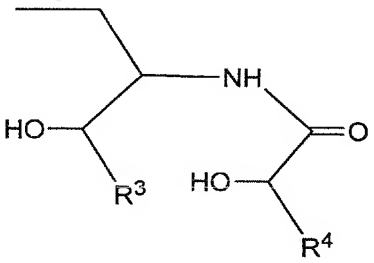
[Formula 11]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 12]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

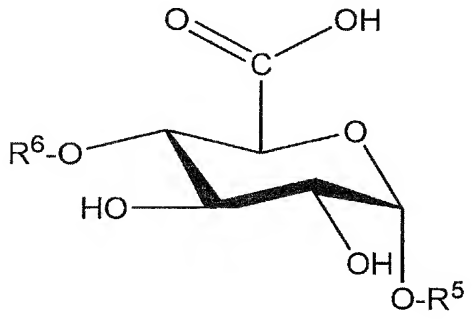
R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.

[Claim 4]

A composition for accelerating IL-4 production comprising a glycosphingolipid having a structure represented by the following formula (3):

formula (3)

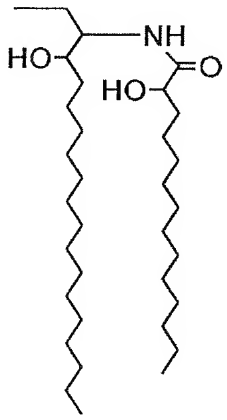
[Formula 13]



wherein R^5 represents R^{51}, R^{52}, or R^{53}; and R^6 represents hydrogen, R^{62}, R^{63}, R^{64}, or R^{65}:

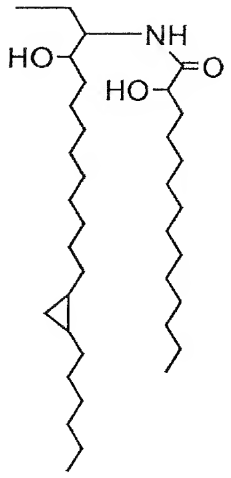
R^{51}:

[Formula 14]



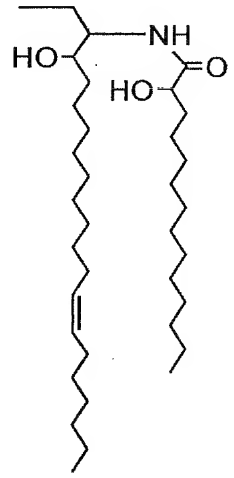
R^{52}:

[Formula 15]



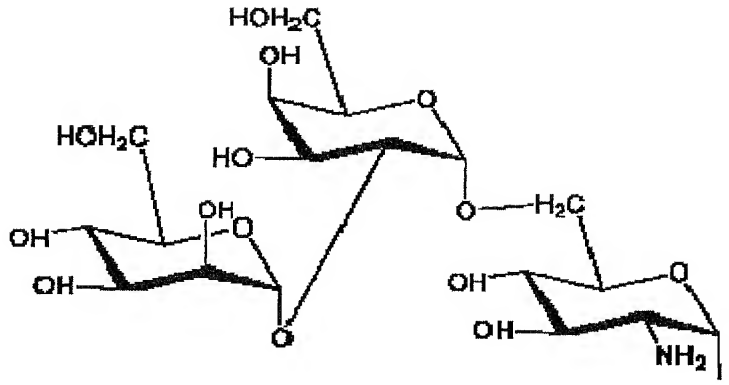
R^{53}:

[Formula 16]



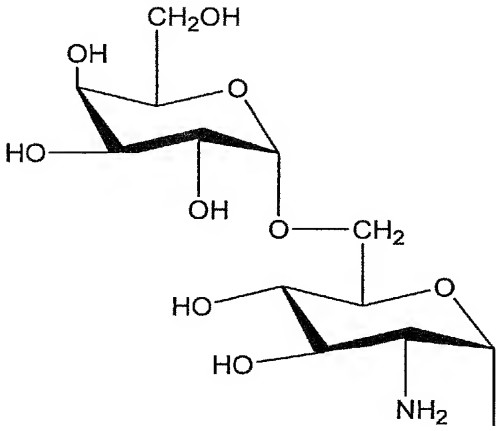
R^{62}:

[Formula 17]



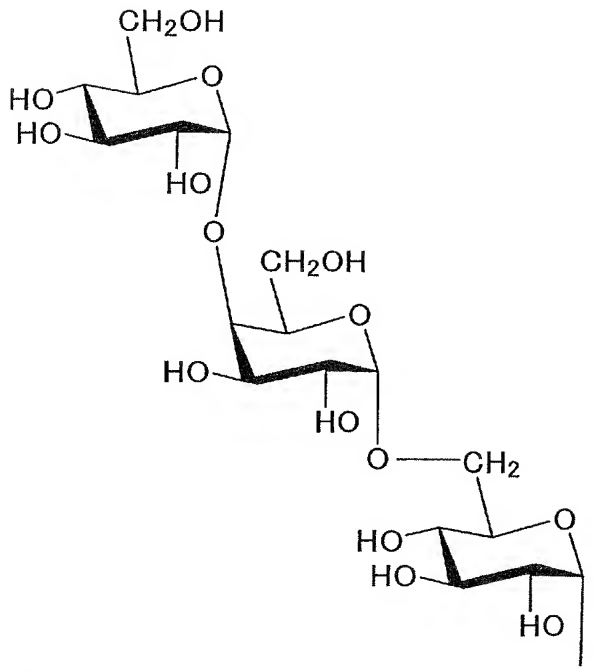
R^{63}:

[Formula 18]



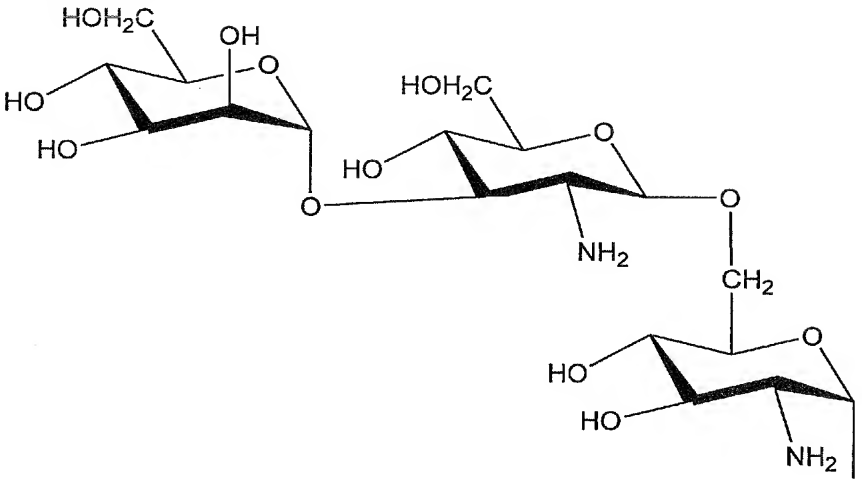
R^{64}:

[Formula 19]



R^{65}:

[Formula 20]

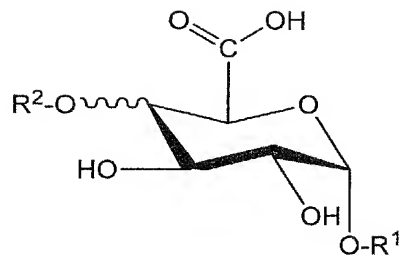


[Claim 5]

A composition for accelerating IFN-γ production comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

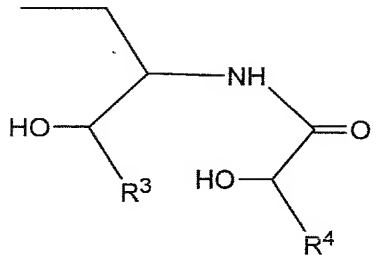
[Formula 21]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 22]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

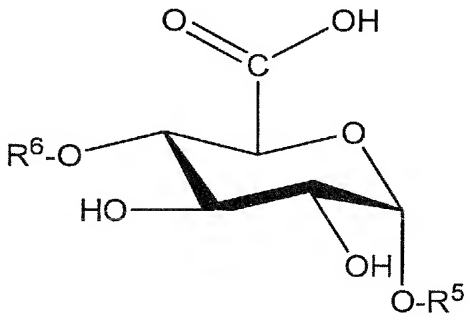
R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.

[Claim 6]

A composition for accelerating IFN-γ production comprising a glycosphingolipid having a structure represented by the following formula (3):

formula 3

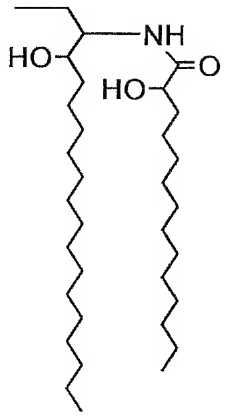
[Formula 23]



wherein R^5 represents R^{51}, R^{52}, or R^{53}; and R^6 represents hydrogen, R^{62}, R^{63}, R^{64}, or R^{65}:

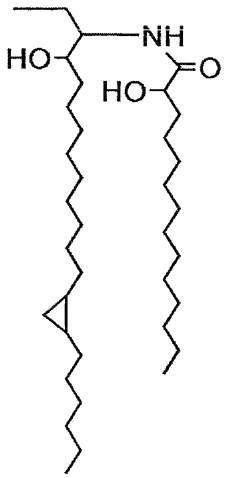
R^{51}:

[Formula 24]



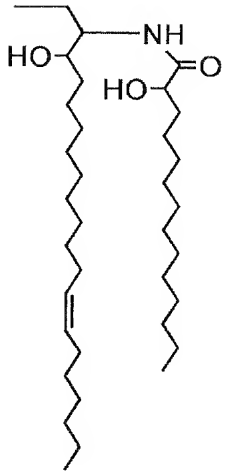
R^{52}:

[Formula 25]



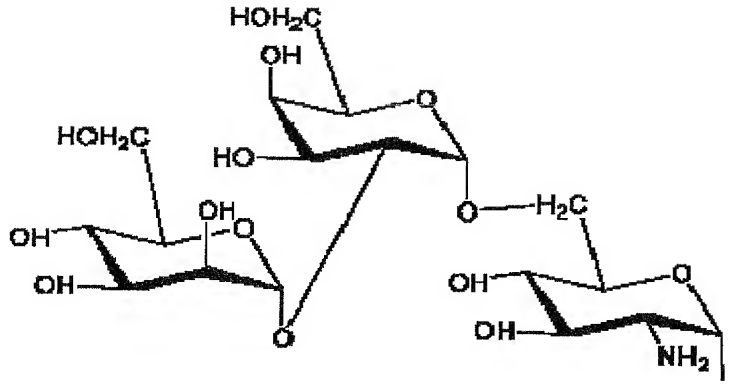
R^{53}:

[Formula 26]



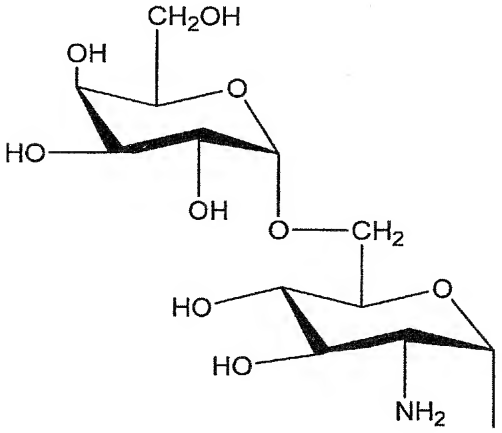
R^{62}:

[Formula 27]



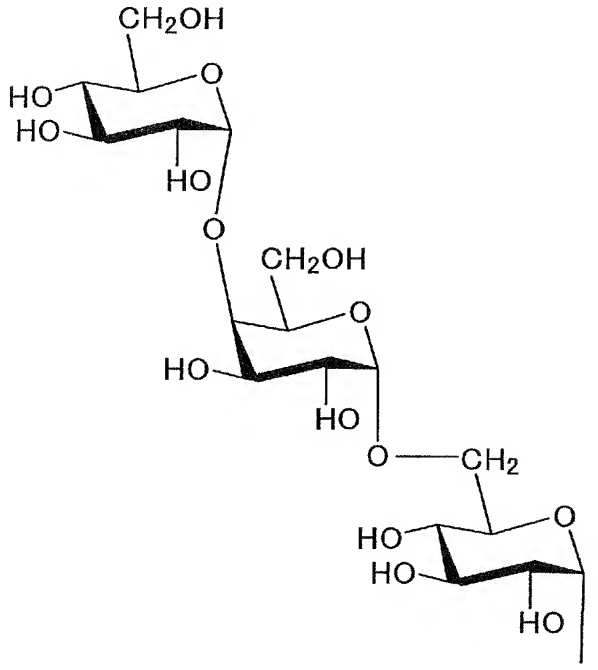
R^{63}:

[Formula 28]



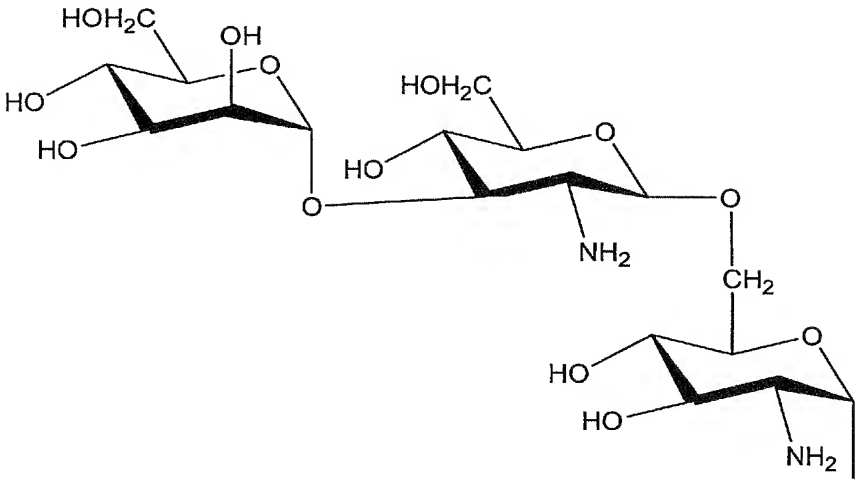
R^{64}:

[Formula 29]



R^{65}:

[Formula 30]



[Document Name] Description

[Title of the Invention] A composition for NKT cell activation, a composition for accelerating IL-4 production, and a composition for accelerating IFN-γ production

[Field of the Invention]

[0001]

The present invention relates to a composition for NKT cell activation, a composition for accelerating IL-4 production, and a composition for accelerating IFN-γ production.

[Background art]

[0002]

It has been heretofore considered that glycosphingolipids are present on the surface layers of animal cells or the like and that they are associated with the recognition mechanism. In contrast, gram-negative bacteria have outer membranes consisting of lipopolysaccharides, proteins, and phosphoric acids, on their cell cortexes, and they interact with the outer world via the outer membranes. Accordingly, lipopolysaccharides as the primary components of the outer membranes had been considered to be present in and to be essential for all gram-negative bacteria. In recent years, however, it has become known that the aerobic gram-negative bacteria *Sphingomonas paucimobilis*, previously known as "*Pseudomonas paucimobilis*," do not comprise lipopolysaccharides and comprise glycosphingolipids as bacterial lipids.

[0002]

The present inventors have succeeded in isolating the glycolipids from the aforementioned *Sphingomonas paucimobilis*, analyzing the chemical structure thereof, and identifying the same (Patent document 1). Also, the present inventors have disclosed that the aforementioned glycosphingolipids have excellent moisturizing effects and barrier effects and thus are extensively applicable as cosmetics (Patent document 2). Further, the present inventors have elucidated the fact that the aforementioned glycosphingolipids have excellent emulsifying effects (Patent document 3).

[0004]

The present inventors have also disclosed that glycosphingolipids obtained from bacterial strains of the other genus *Sphingomonas* are excellent as cosmetic and pharmaceutical

compositions (Patent Document 4).

[0005]

It has been reported that the NKT cells expressing T cell receptors (TCR) are related to NK cells in the following respect: they exhibit large granular lymphocyte-like (LGL-like) in morphologies, they constantly express the IL-2R β chain, and they have perfolin. However, they are completely different from NK cells in terms of the possession of TCR (Non-patent Document 1).

Given the circumstances, it has been reported that mouse NKT cells that express NK1.1 in IL-12-activated T cells are important effector cells for inhibiting hematogenous metastasis of tumors to the liver or lung (Non-patent Document 2, Non-patent Document 3).

As described above, the NKT cells have drawn attention as a new group of cells in recent years.

[0006]

Further, Patent Document 5 describes that α-glycosylceramide having a given structure is effective as an NKT cell activator. A more effective NKT cell activator has been desired.

[0007]

[Patent Document 1] WO 92/12986

[Patent Document 2] JP Patent Publication No. 11-43437

[Patent Document 3] JP Patent Publication No. 2000-51676

[Patent Document 4] JP Patent Publication No. 2002-010797

[Patent Document 5] WO 98/44928

[Non-patent Document 1] J. Immunol., 155, 2972, 1995

[Non-patent Document 2] J. Immunol., 154, 4333, 1995

[Non-patent Document 3] J. Immunol., 88, 82, 1996

[Disclosure of the invention]

[Problems to be solved by the Invention]

[0008]

An object of the present invention is to overcome the aforementioned problems and provide more effective for NKT cell activator. The object of the present invention is to provide

a composition for accelerating IL-4 production, and a composition for accelerating IFN-γ production.

[Means for solving the Problems]

[0009]

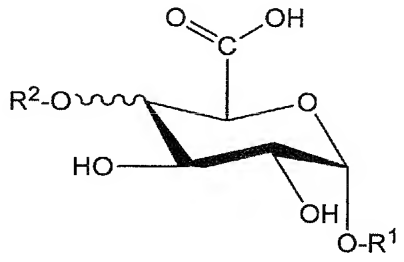
Under the above circumstances, the present inventors found that the object could be attained by the following means.

[0010]

(1) A composition for NK cell activation comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

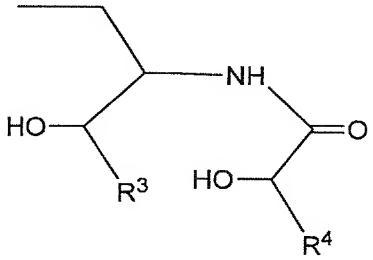
[Formula 1]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 2]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

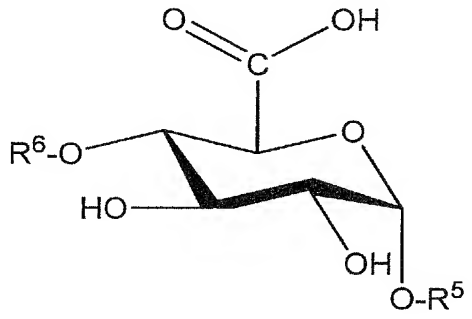
R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.

[0011]

(2) A composition for NKT cell activation comprising a glycosphingolipid having a structure represented by the following formula (3):

formula (3)

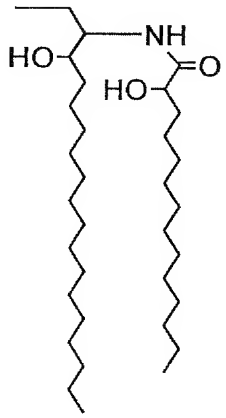
[Formula 3]



wherein R^5 represents R^{51}, R^{52}, or R^{53}; and R^6 represents hydrogen, R^{62}, R^{63}, R^{64}, or R^{65}:

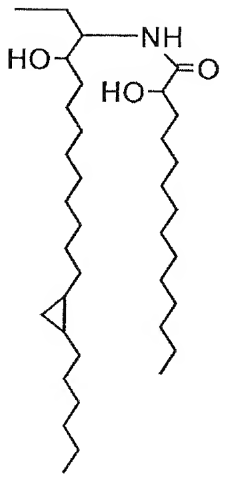
R^{51}:

[Formula 4]



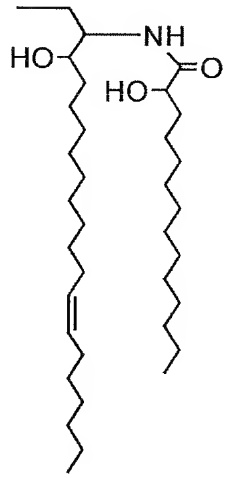
R^{52}:

[Formula 5]



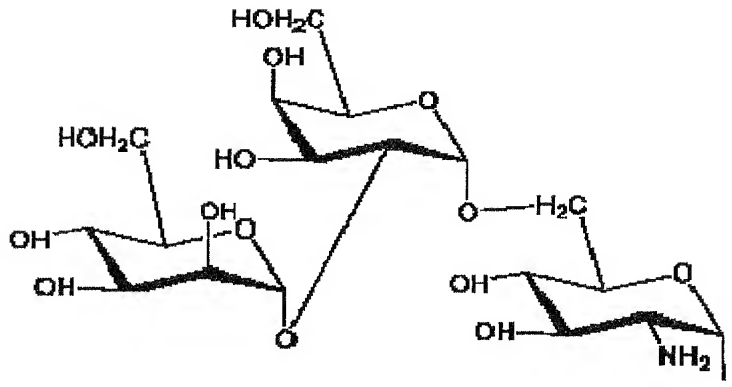
R^{53}:

[Formula 6]



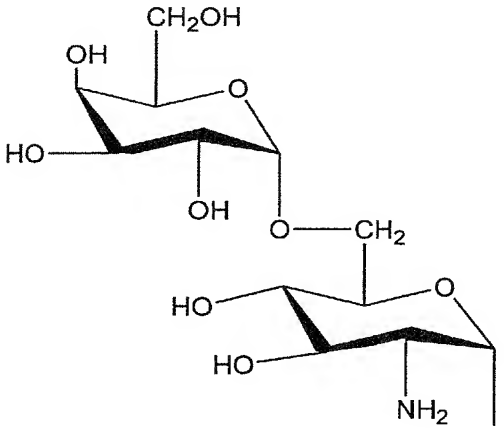
R^{62}:

[Formula 7]



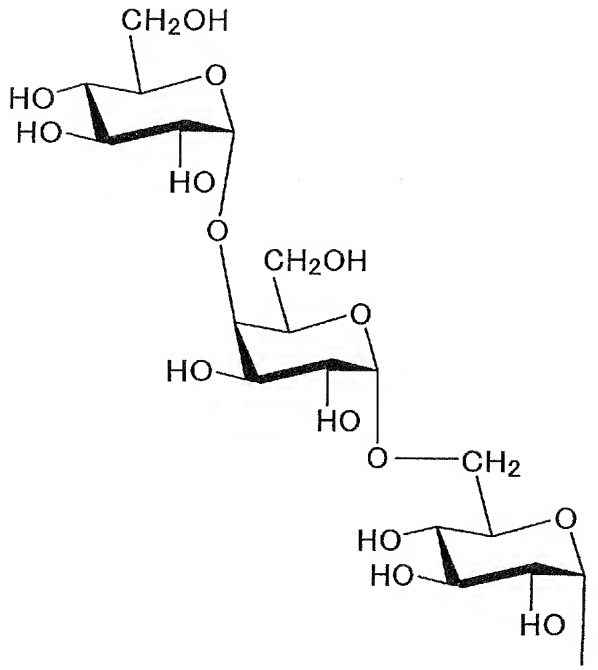
R^{63}:

[Formula 8]



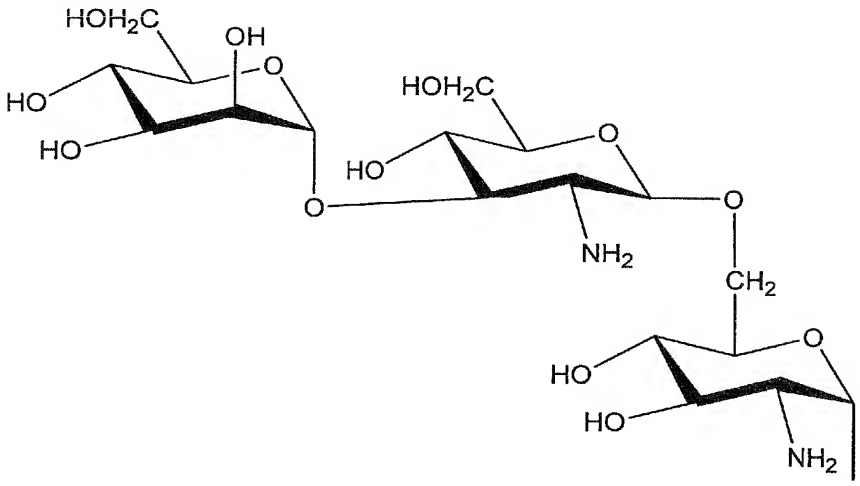
R^{64}:

[Formula 9]



R^{65}:

[Formula 10]

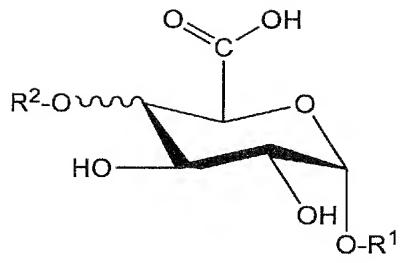


[0012]

(3) A composition for accelerating IL-4 production comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

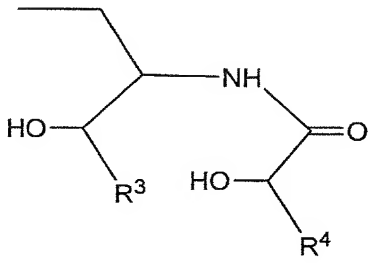
[Formula 11]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 12]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

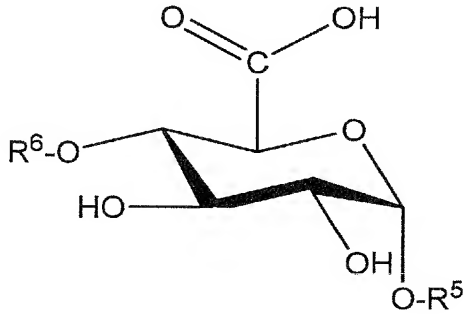
R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.

[0013]

(4) A composition for accelerating IL-4 production comprising a glycosphingolipid having a structure represented by the following formula (3):

formula (3)

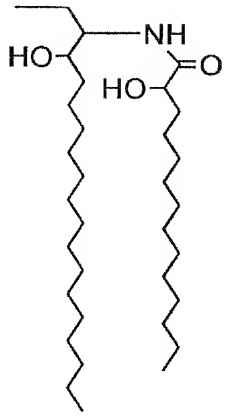
[Formula 13]



wherein R^5 represents R^{51}, R^{52}, or R^{53}; and R^6 represents hydrogen, R^{62}, R^{63}, R^{64}, or R^{65}:

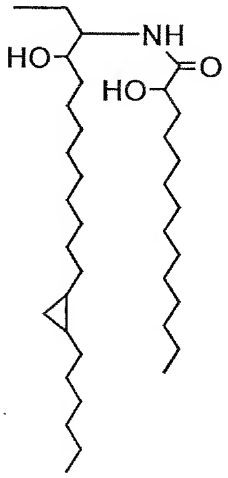
R^{51}:

[Formula 14]



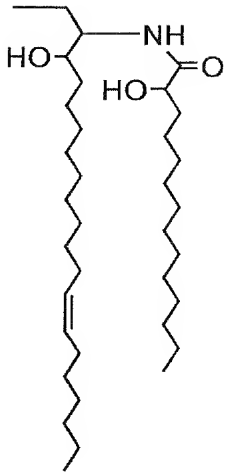
R^{52}:

[Formula 15]



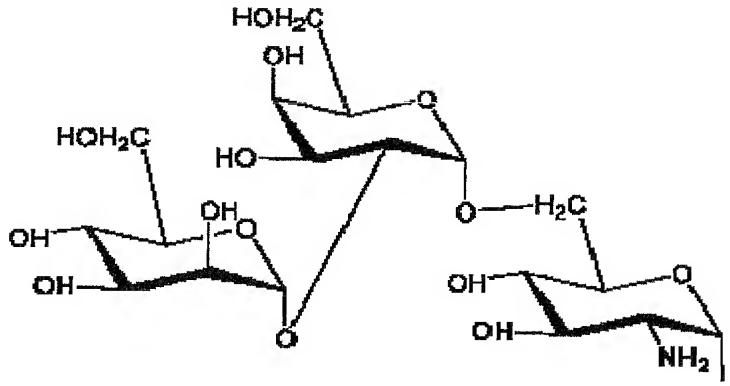
R^{53}:

[Formula 16]



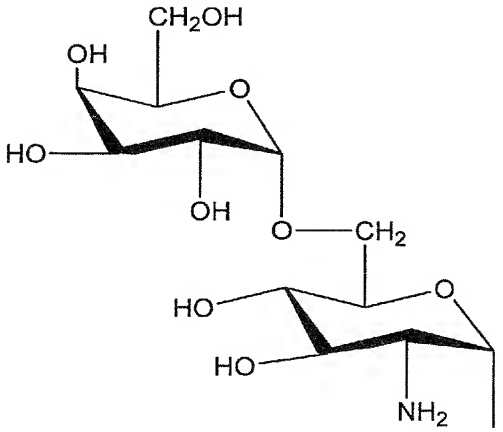
R^{62}:

[Formula 17]



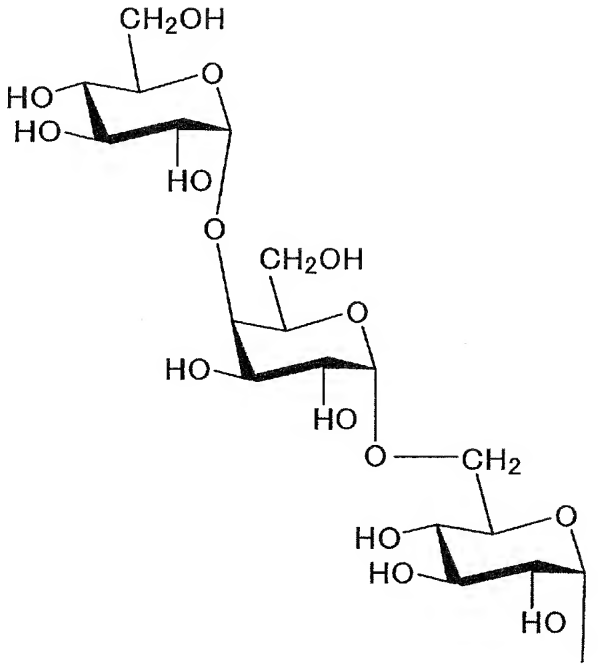
R^{63}:

[Formula 18]



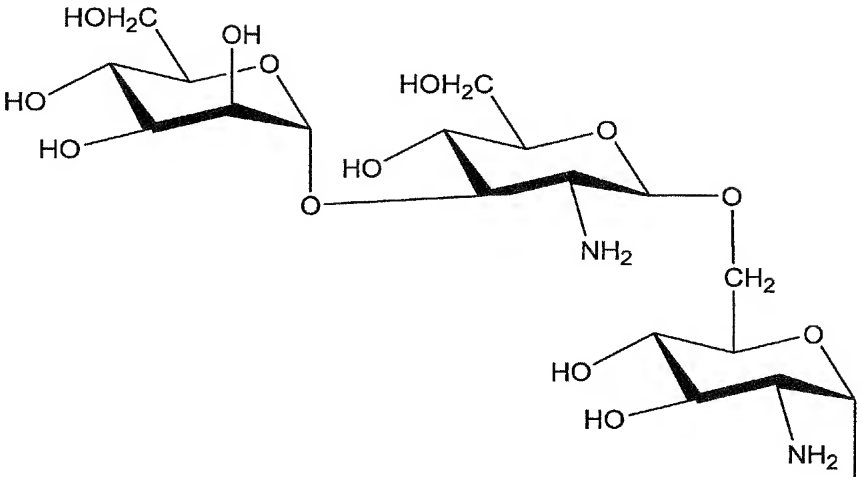
R^{64}:

[Formula 19]



R^{65}:

[Formula 20]

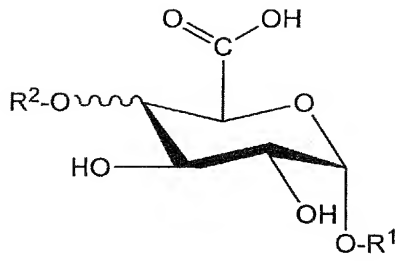


[0014]

(5) A composition for accelerating IFN-γ production comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

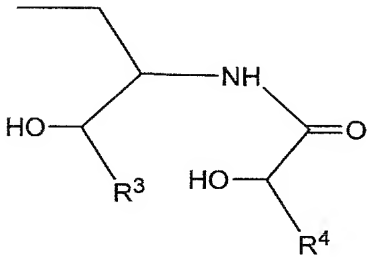
[Formula 21]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 22]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

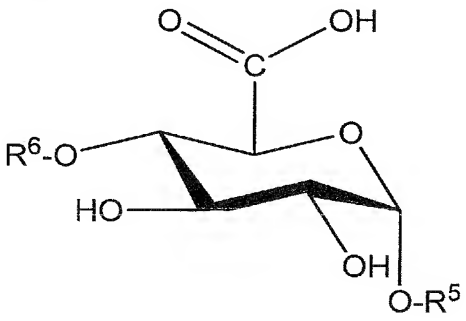
R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.

[0015]

(6) A composition for accelerating IFN-γ production comprising a glycosphingolipid having a structure represented by the following formula (3):

formula (3)

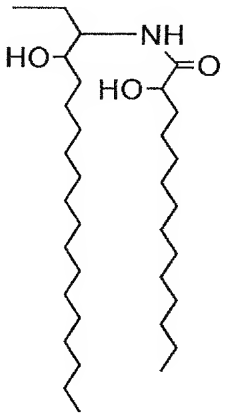
[Formula 23]



wherein R^5 represents R^{51}, R^{52}, or R^{53}; and R^6 represents hydrogen, R^{62}, R^{63}, R^{64}, or R^{65}:

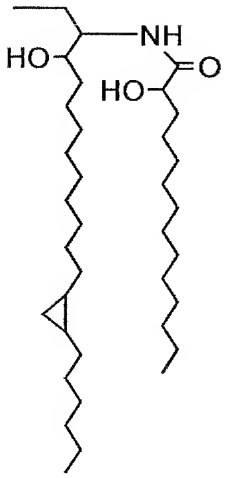
R^{51}:

[Formula 24]



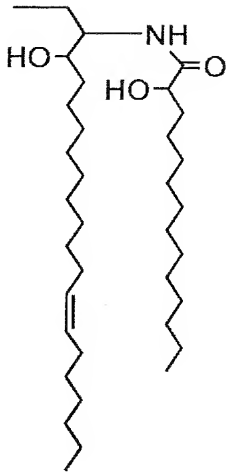
R^{52}:

[Formula 25]



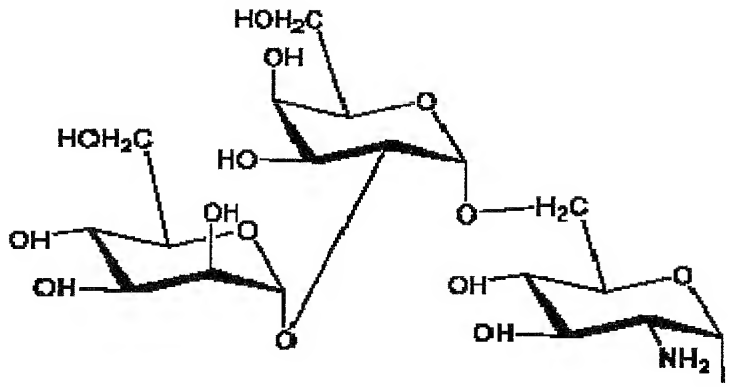
R^{53}:

[Formula 26]



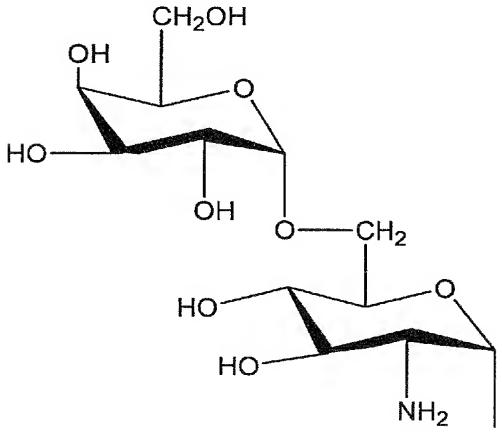
R^{62}:

[Formula 27]



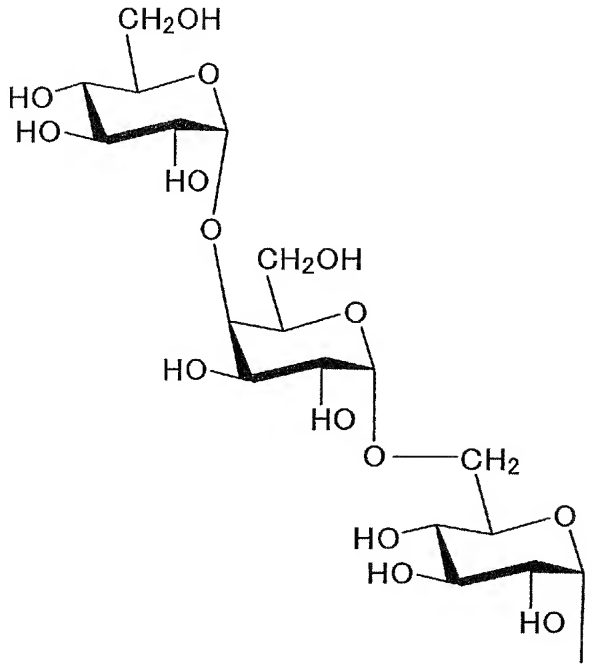
R^{63}:

[Formula 28]



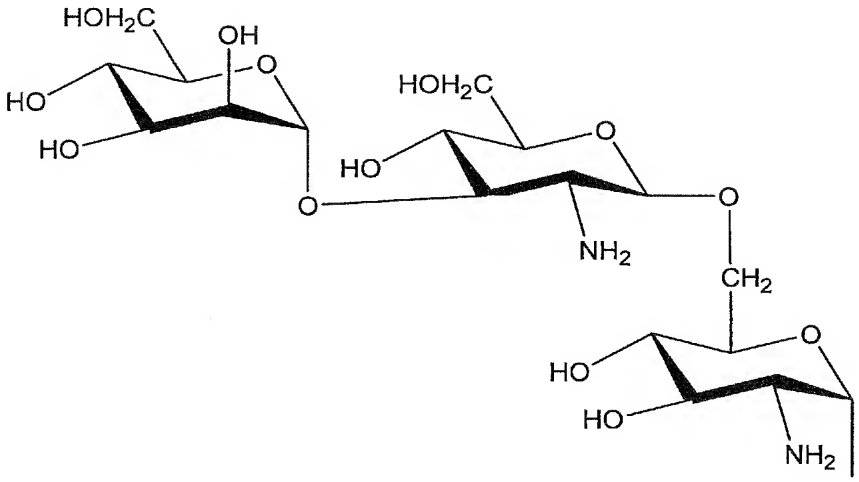
R^{64}:

[Formula 29]



R^{65}:

[Formula 30]



[Advantageous effects of the invention]

[0016]

The present invention provides a more effective composition for NKT cell activation. In addition, a composition for more effectively accelerating IL-4 production and / or IFN-γ production was obtained.

[Embodiments for carrying out the Invention]

[0017]

Hereafter, the present invention is described in detail. In the present specification, the symbol "~" is used to denote the numerical values that precede or follow the same as lower limits or upper limits.

[0018]

The glycosphingolipid that is used in the present invention have the structure represented by the formula (1). Cycloalkyl which may have alkyl represented by R^3 in formula (1) at the alkyl terminus or in the alkyl chain. A preferable example of cycloalkyl is cyclopropane. Alkyl represented by R^3 has preferably 13 to 23, and more preferably 15, 16, 17, 18, 19, 20 or 21, carbon atoms. Alkyl or alkenyl represented by R^3 preferably has a substituted or unsubstituted straight chain. A double bond may be present at any position in alkenyl.

[0019]

In contrast, alkyl represented by R^4 has preferably 10 to 20 carbon atoms, more preferably 10, 11, 12, 13, 14, or 15 carbon atoms. Alkyl represented by R^4 preferably has a substituted or unsubstituted straight chain.

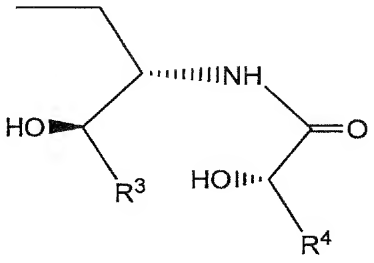
More preferably, R^1 is any of the aforementioned R^{51} ~ R^{53}.

[0020]

R^1 in formula (1) preferably represents the conformation represented by the following formula (1-2):

formula (1-2)

[Formula 31]



wherein R^3 and R^4 are as defined above in formula (1-1). Thus, the aforementioned R^{51} ~ R^{53} having such conformations are more preferable.

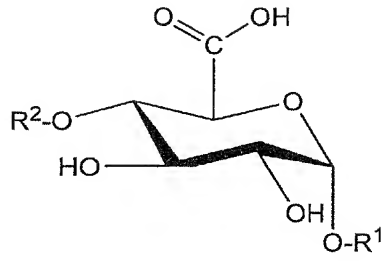
[0021]

Formula (1) is preferably represented by the formula (2), (3), (4), or (5). formula (2)

is as shown below:

formula (2)

[Formula 32]



wherein R^1 and R^2 are as defined above in formula (1), and their preferable ranges are also as defined above.

[0022]

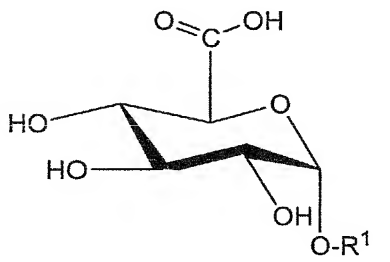
In a preferable structure represented by the formula (3), R^5 is any of R^{51}, R^{52}, or R^{53}, and R^6 is hydrogen (hereafter the same may be referred to as "Structure A"), R^5 is any of R^{51}, R^{52}, or R^{53}, and R^6 is R^{62} (hereafter the same may be referred to as "Structure B"), R^5 is any of R^{51}, R^{52}, or R^{53}, and R^6 is R^{63} (hereafter the same may be referred to as "Structure C"), R^5 is any of R^{51}, R^{52}, or R^{53}, and R^6 is R^{64} (hereafter the same may be referred to as "Structure D"), or R^5 is any of R^{51}, R^{52}, or R^{53}, and R^6 is R^{65} (hereafter the same may be referred to as "Structure E"). Further, Structure A in which R^5 is R^{52}, or R^{53}, is more preferably used.

[0023]

Formula (4) is as shown below:

formula (4)

[Formula 33]



wherein R^1 is as defined above in formula (1), and its preferable range is also as defined above

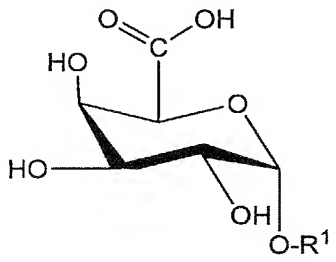
(a composition comprising at least one compound represented by the formula (4) may be hereafter referred to as "Structure AA").

[0024]

Formula (5) is as shown below:

formula (5)

[Formula 34]



wherein R^1 is as defined above in formula (1) (hereafter the same may be referred to as "Structure F"), and its preferable range is also as defined above.

[0025]

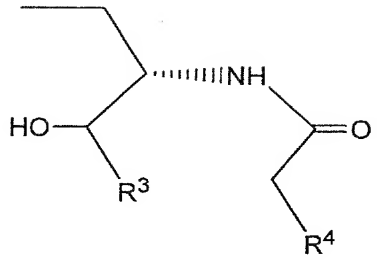
In the present invention, a single type or two or more types of glycosphingolipids may be used. When two or more types of glycosphingolipids are used in combination, the proportion of each component is not particularly limited. For example, a composition comprising at least one of the 3 types of compounds having Structure A, a composition comprising at least one of the 3 types of compounds having Structure B, or a composition comprising at least one of the compounds having Structure F may be used. Among them, a structure in which R^1 is any of R^{51}, R^{52}, or R^{53} is preferable (hereafter the same may be referred to as "Structure FA").

[0026]

In addition to the composition described above, the composition disclosed in the present invention may include composition represented by the formula (1) wherein formula (1-1) is represented by the conformation represented by the formula (1-1-1):

formula (1-1-1)

[Formula 35]



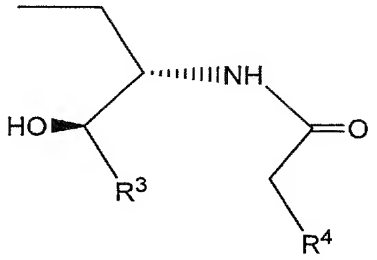
wherein R^3 and R^4 are as defined above in formula (1-1), and their preferable ranges are also as defined above.

[0027]

Formula (1-1-1) further preferably has the structure represented by the formula (1-1-2):

formula (1-1-2)

[Formula 36]



wherein R^3 and R^4 are as defined above in the formula (1-1), and their preferable ranges are also as defined above.

[0028]

The glycosphingolipid represented by the formula (1) can be extracted from bacteria having glycosphingolipid. For example, methods disclosed in WO 92/12986 or JP Patent Publication No. 2002-10797 can be adopted. Since glycosphingolipid is contained in bacteria of the genus *Sphingomonas*, the glycosphingolipid represented by the formula (1) can be extracted from any of the bacteria of the genus *Sphingomonas*. The bacteria of the genus *Sphingomonas* include those that have been generally said to belong to the genus *Sphingomonas* and those that are classified as belonging to substantially the same genus as the genus *Sphingomonas*. For example, any bacteria described in Microbiol. Immunol., 2000, 44, 563-575 can be used in the present invention.

The glycosphingolipids represented by the formula (1) are insoluble in acetone. Accordingly, bacteria are preferably washed with acetone before extraction. An alcoholic solvent such as methanol or a mixed solvent comprising an alcoholic solvent mixed with a polar solvent such as chloroform is preferably used for extraction of the glycosphingolipid represented by the formula (1) from the viewpoint of yield. Other types of solvents may be used as long as such solvents are capable of dissolving glycosphingolipid.

[0029]

When a mixture of glycosphingolipids is obtained, components thereof can be separated from one another in accordance with a technique known in the art. For example, glycosphingolipids can be completely separated via chromatography. When a mixed solution of chloroform and methanol is used as an eluate, glycosphingolipids of Structure A, Structure F, Structure C, and Structure B or Structure D or Structure E, are eluted in that order. Since Structure B, Structure D, and Structure E are generally produced by different bacteria, glycosphingolipids can be very easily separated. Conditions for separation via chromatography, such as a filler, an eluate, a rate of elution, pressure, and temperature, can be adequately regulated. Alternatively, a reagent that selectively reacts with a specific substance contained in the glycosphingolipid mixtures may be used in order to prepare a derivative of such substance, and separation can be carried out with the utilization of chemical or physical properties of such derivative. When the bacterium *Sphingomonas paucimobilis* is used, glycosphingolipids of Structure A and of Structure B are generally obtained. When the bacterium *Sphingomonas capsulata* (nomen novum: *Novosphingobium capsulatum*) is used, glycosphingolipids of Structure A and of Structure C are generally obtained. When the bacterium *Sphingomonas adhaesiva* is used, glycosphingolipids of Structure A and of Structure D are generally obtained. When *Sphingomonas* sp. MK346 is used, glycosphingolipids of Structure A and of Structure E are generally obtained. When *Sphingomonas wittichii*, *Sphingomonas macrogoltabidus* (nomen novum: *Sphingopyxis macrogoltabida*), *Sphingomonas terrae*, or *Sphingomonas yanoikuyae* (nomen novum: *Sphingobium yanoikuyae*) is used, glycosphingolipids of Structure AA (e.g., Structure A) and of Structure F (e.g., Structure FA) are generally obtained. Accordingly, glycosphingolipids of interest can be efficiently obtained by selecting bacteria based on such

information.

[0030]

The glycosphingolipid represented by the formula (1) can be also synthesized by combining conventional synthesis techniques. For example, a sugar and a sphingosine portion are first synthesized or extracted from the bacteria. Then, the glycosphingolipid represented by the formula (1) can be prepared by forming amide bonds between the sugar and the sphingosine portion.

[0031]

In the present invention, NKT cells may include, for example, human $V\alpha24^+$ NKT cells and mouse $V\alpha14^+$ NKT cells. NKT cell activation may include enhancement of cytotoxic activity, enhancement of cytokine production, and acceleration of NKT cell proliferation. Further, the composition for NKT cell activation of the present invention accelerates the production of IL-4 and IFN-γ as a consequence. Accordingly, the composition disclosed in the present invention can be used as accelerators of various functions accelerated by IL-4 or IFN-γ. Examples of functions accelerated by IL-4 may include Th2 induction and induction of antibody class switch. Examples of functions accelerated by IFN-γ may include Th1 induction and macrophage activation.

In addition to the aforementioned, the compositions for accelerating production or activation of each type of IL, IFN-α, IFN-β, tumor necrosis factors (TNF), lymphotoxin, hematopoietic colony-stimulating factors (CSF), erythropoietin, hematopoietic epidermal growth factors (EGF), and fibroblast growth factors (FGF) can be used as enhancement of cytokine. Further, the glycosphingolipids disclosed in the present invention can also be used as the other immune-activating composition, an apoptosis-inducing composition for cancer cells, or a composition for accelerating production or activation targeting acceleration of NF-kappaB activation, I-kappaB degradation, p38 phosphorylation, or Akt phosphorylation.

[0032]

When the composition of the present invention is used as pharmaceutical preparations, quasi-drugs, or active ingredients thereof, the composition can be preferably administered as a pharmaceutical composition that can be produced by a method known in the art. Examples of

pharmaceutical composition may include tablets, capsules, powders, subtle granules, granules, liquids, and syrups. Such pharmaceutical compositions can be produced with the addition of pharmacologically or pharmaceutically acceptable additives. Examples of pharmacologically or pharmaceutically acceptable additives include vehicles, disintegrators or disintegration assistants, binders, lubricants, coating agents, dyes, diluents, bases, solubilizers or disintegration assistants, isotonizing agents, pH regulators, stabilizers, propellants, and adhesives. The pharmaceutical composition may also comprise one or more other composition for NKT cell activation without departing from the scope of this invention. The dose of the pharmaceutical preparations according to the present invention is not particularly limited, and it can be adequately determined in accordance with the type of active ingredient or other conditions. Such dose can be adequately increased or decreased in accordance with a variety of general factors such as the body weight or age of the patient, the type or symptom of the disease, or the route of administration. In general, the pharmaceutical composition can be administered in amounts of 0.001 mg to 100 mg, and preferably 0.01 mg to 10 mg, per adult per day. The route of administration is not particularly limited, and administration can be made intravenously via injection or infusion or orally.

[Example]

[0033]

The present invention is hereafter described in greater detail with reference to the examples. The materials, the amounts used, the percentages, the procedures, the processes, and other conditions described in the following examples can be adequately altered without departing from the scope of this invention. Accordingly, the technical scope of the present invention is not limited to the examples.

[0034]

Assay of activity of NKT cell activation

Glycosphingolipid : A produced from *Sphingomonas paucimobilis* (GSL-1), Structure B produced from *Sphingomonas paucimobilis* (GSL-2), Structure AA produced from *Sphingomonas yanoikuyae* (GSL-6), and Structure F produced from *Sphingomonas yanoikuyae* (GSL-7), were used in the examples.

[0035]

Animals :

C57BL/10ScSn mice (hereafter referred to as "normal mice") and C57BL/10ScCr mice (TLR4-deficient/IL-12Rβ2 chain mutant mice) (hereafter referred to as "TLR4-deficient mice;" 7-week-old, female, the Max-Planck Institute of Immunobiology, Freiburg, Germany) were used as test animals. GSL-1 and GSL-2 activate macrophages of normal mice via TLR4 and induce IL-12 production. TLR4-deficient mice have mutation in the β-chain of the IL-12 receptor and thus IL-12 does not act. IL-12 is a substance that potently activates the immune system. With the use of the TLR4-deficient mice of the present example, acceleration of NKT cell activation by the glycosphingolipid that are used in the present invention can be further clarified without the influence of IL-12 activity.

[0036]

Preparation of samples:

The GSLs (10 μg) was administered to normal mice and to TLR4-deficient mice. Administration was carried out via caudal veins. Then, the serum and the liver were extracted from the mice, one of which had been administered physiological saline as a control (the control), another of which is one day after the administration (day 1), and the other of which is two days after the administration (day 2).

[0037]

Separation of intrahepatic leukocytes:

The sampled liver was crushed using 2 glass slides. A cell suspension was centrifuged at 500 rpm for 1 minute. The obtained supernatant was centrifuged at 1,200 rpm (300 g) for 5 minutes. The resulting sediment was suspended in 30% Percoll (Pharmacia), and the resulting suspension was superposed on 67.5% Percoll, centrifugation was carried out at 20°C at 2,000 rpm (800 g) for 30 minutes, and leukocytes were accumulated at the boundary of 30% Percoll and 67.5% Percoll and then collected. The obtained cells were washed three times with the Hanks medium to obtain intrahepatic leukocytes.

[0038]

Flow cytometry:

In order to block nonspecific binding of a fluorescently labeled antibody through FcR, anti-FcγR (2.4G2) was used. The PE-labeled anti-NK1.1 antibodies (PK136, Nippon Becton Dickinson Co., Ltd.) and the biotin-labeled anti-TCRαβ antibodies (H57-597, Nippon Becton Dickinson Co., Ltd.) were used. After the antibodies were added to the cells, the reaction was allowed to proceed at 4°C in the dark for 30 minutes. The cells treated with the biotin-labeled antibodies were allowed to react with Cy-chrome-bound streptavidin (554062, Nippon Becton Dickinson Co., Ltd.) at 4°C in the dark for 30 minutes. Staining of the aforementioned antibodies and Cy-chrome-bound streptavidin and washing of cells were carried out with the use of 0.1% NaN_3-containing 1% serum albumin. The cells were stained and then immobilized with 1% paraformaldehyde-containing phosphate buffered saline(PBS(-)). Assay was carried out using an automated cell sorter (Coulter Epics Elite ESP, Beckman Coulter). This procedure was separately carried out using the FITC-labeled anti-CD11a antibodies (M17/4, Nippon Becton Dickinson Co., Ltd.) instead of the PE-labeled anti-NK1.1 antibodies.

[0039]

The flow cytometry was carried out by the method using the PE-labeled anti-NK1.1 antibodies (or CD11a). The results thereof are shown in Figs 1 to 6. Figs. 1 to 4 sequentially show the cases where GSL-1, GSL-2, GSL-6, and GSL-7 were administered to normal mice. Figs. 5 and 6 sequentially show the cases where GSL-1 and GSL-2 were administered to TLR4-deficient mice.

In the drawings, numerical values represent the percentages (%) of cells wherein both NK1.1 (or CD11a) and TCRαβ were expressed. It should be noted that the cells wherein both NK1.1 (or CD11a) and TCRαβ were expressed are NKT cells.

[0040]

The flow cytometry for normal mice was carried out in the same manner as described above by the method using the PE-labeled anti-NK1.1 antibodies on a different day. The results thereof (the percentages of NKT cells) are shown in Fig. 7. In the drawing, numerical values represent the percentages (%) of cells wherein both NK1.1 (or CD11a) and TCRαβ were expressed, and the reference marks represent the cells that were found to be statistically significantly different from the control as a result of the t-test.

[0041]

Confirmation of the presence of IFN-γ:

The IFN-γ content in the blood after GSL-1 or GSL-2 had been administered was examined. GSL-1 or GSL-2 was administered to the normal mice and to the TLR4-deficient mice in the same manner as described above, and the IFN-γ content in the serum was analyzed.

The IFN-γ content was analyzed by the sandwich ELISA method using the purified anti-IFN-γ antibodies (R4-6A2, Nippon Becton Dickinson Co., Ltd.) and the biotin-conjugated anti-IFN-γ antibodies (AN-18, detection antibodies, Nippon Becton Dickinson Co., Ltd.) as a detectable antibody. After the reaction using the biotin-conjugated antibodies, alkaline phosphatase-labeled streptavidin (43-4822, Zymed) was conjugated thereto, and the color was developed using paranitrophenyl phosphate (N-4645, Sigma) as a substrate. The absorbance at 405 nm and that at 540 nm as the control were measured using a microplate reader (Model: 550, Nippon BioRad Laboratories). As a result, IFN-γ production was found to be accelerated in the normal mice, as shown in Fig. 8. Even though GSL-1 or GSL-2 was administered to the TLR4-deficient mice, IFN-γ was not detected because they did not react with IL-12.

[0042]

Flow cytometry of IFN-γ-producing NKT cells:

The normal mice to which GSL-1 or GSL-2 had been administered were subjected to flow cytometry in the same manner as described above, cells were stained with the PE-labeled anti-NK1.1 antibodies, the biotin-labeled anti-TCRαβ antibodies, and the FITC-labeled anti-IFN-γ antibodies (554410, Nippon Becton Dickinson Co., Ltd.), and the percentage of IFN-γ-producing NKT cells was determined. The results are shown in Fig. 9. In the drawing, numerical values represent the percentages of IFN-γ-producing NKT cells wherein both NK1.1 and TCRαβ were expressed.

[0043]

Measurement of IL-4 content:

Whether or not the IL-4 content in the blood increased after GSL-1 or GSL-2 had been administered was examined. GSL-1 or GSL-2 was administered to the normal mice and to the TLR4-deficient mice in the same manner as described above, and the IL-4 content in the serum

was analyzed.

The IL-4 content was measured by ELISA using the BD Opti-EIA mouse IL-4 set (555232, Nippon Becton Dickinson Co., Ltd.) in accordance with the instruction manual. As a result, increase of IL-4 was observed in every mouse. In particular, IL-4 production was significantly accelerated in the TLR4-deficient mice, as shown in Fig. 10.

[0044]

When GSL-1 or GSL-2 was added, increases in the percentages of cells wherein both NK1.1 and TCRαβ had been expressed were observed via the aforementioned flow cytometry. In the cells with significant increase in such expression levels, production of both IFN-γ and IL-4 was confirmed. This indicates that GSL-1 or GSL-2 is effective for activation of NKT cells.

[Brief description of the drawings]

[0045]

[Fig. 1] Fig. 1 shows the analysis results of flow cytometry when GSL-1 has been administered to a normal mouse.

[Fig. 2] Fig. 2 shows the analysis results of flow cytometry when GSL-2 has been administered to a normal mouse.

[Fig. 3] Fig. 3 shows the analysis results of flow cytometry when GSL-6 has been administered to a normal mouse.

[Fig. 4] Fig. 4 shows the analysis results of flow cytometry when GSL-7 has been administered to a normal mouse.

[Fig. 5] Fig. 5 shows the analysis results of flow cytometry when GSL-1 has been administered to a TLR4-deficient mouse.

[Fig. 6] Fig. 6 shows the analysis results of flow cytometry when GSL-2 has been administered to a TLR4-deficient mouse.

[Fig. 7] Fig. 7 shows NKT cell changes when GSL1 to GSL-4 has been administered to a normal mouse.

[Fig. 8] Fig. 8 shows IFN-γ concentration after GSL-1 or GSL-2 has been administered to a normal mouse.

[Fig. 9] Fig. 9 shows changes in IFN-γ-producing NKT cells when GSL-1 or GSL-2 has been administered to a normal mouse.

[Fig. 10] Fig. 10 shows IL-4 concentration after GSL-1 or GSL-2 has been administered to a TLR4-deficient mouse.

[Document Name] Drawings

Fig. 1

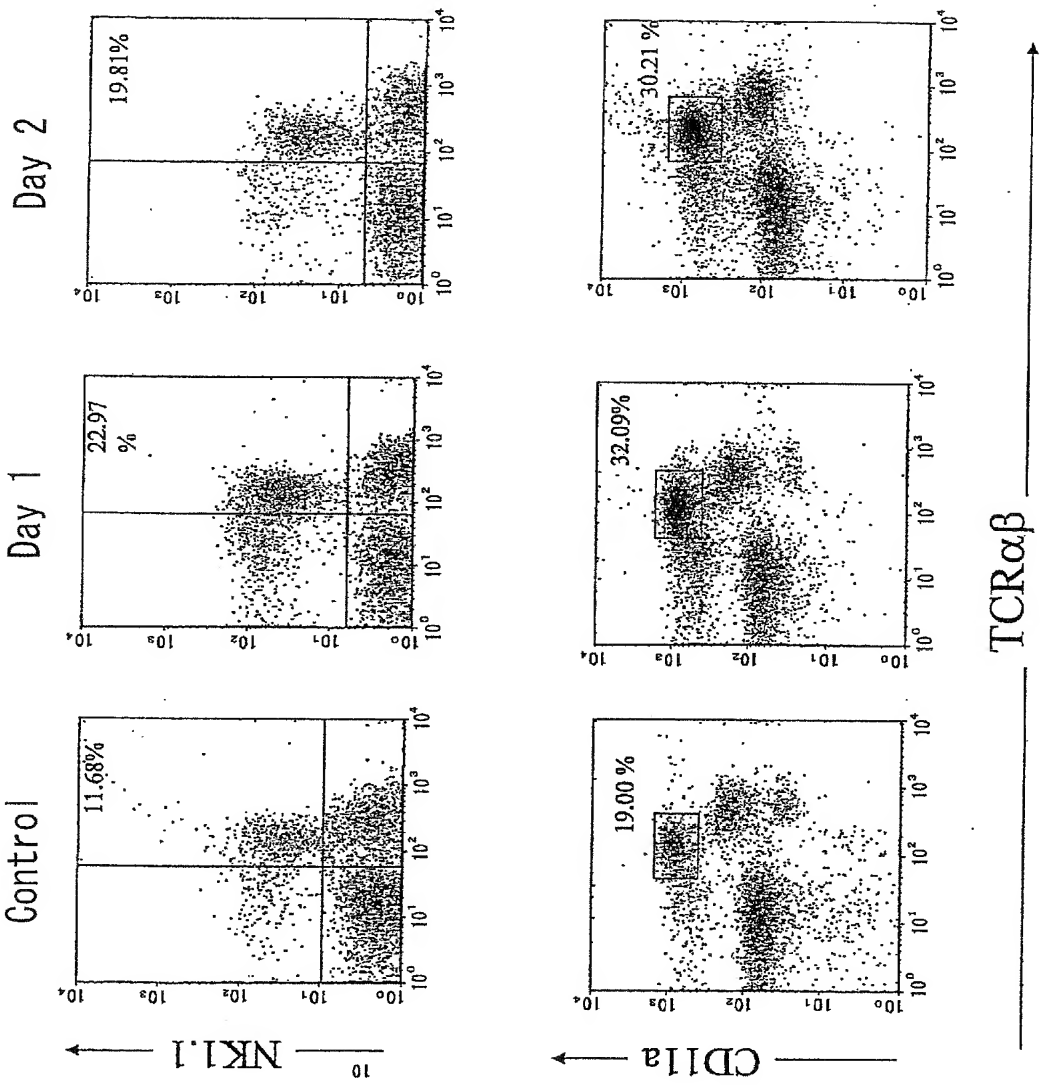


Fig. 2

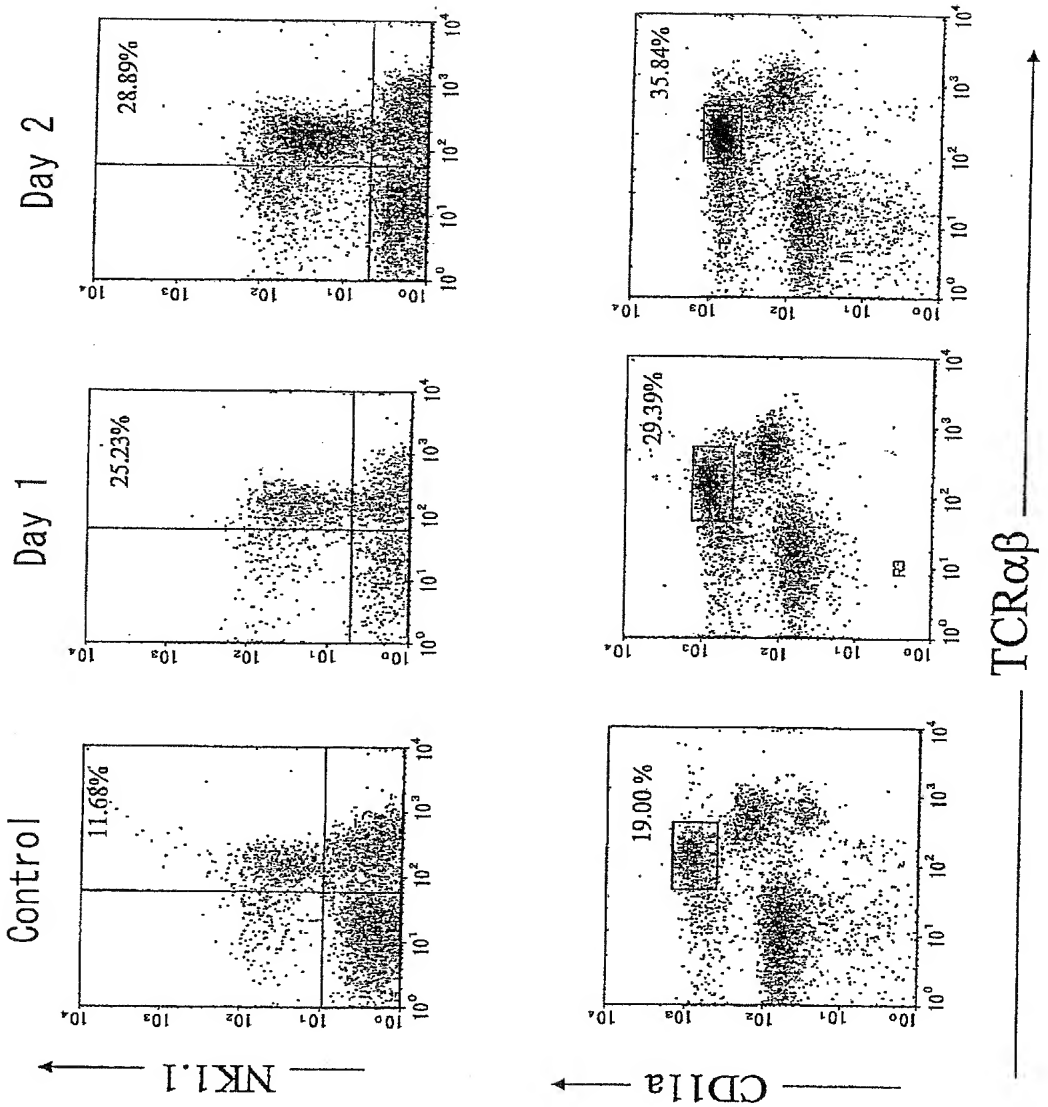

Day 2
Day 1
Control
28.89%
25.23%
11.68%
35.84%
29.39%
19.00 %
NK1.1
CD11a
TCRαβ

Fig. 3

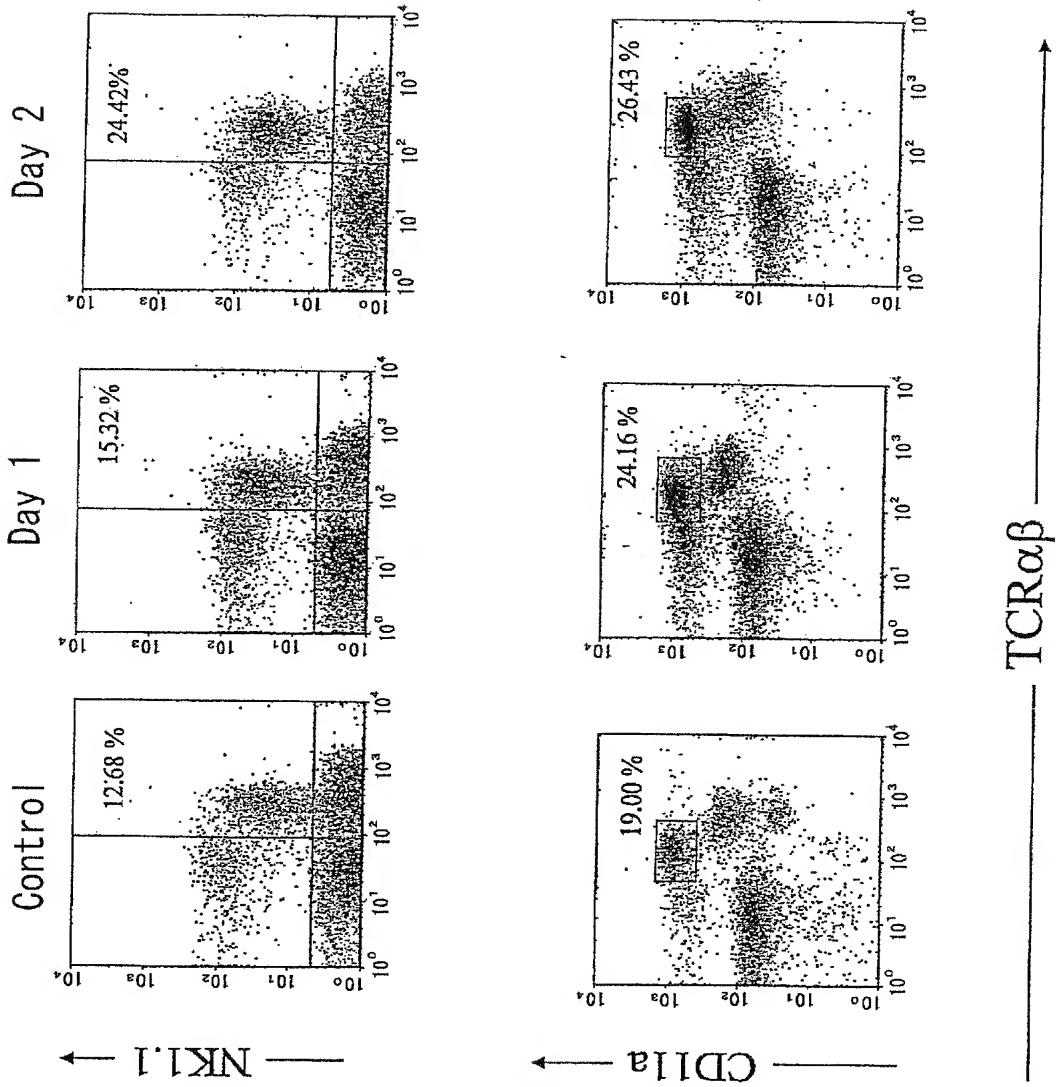

Day 2
Day 1
Control
24.42%
15.32 %
12.68 %
26.43 %
24.16 %
19.00 %
NK1.1
CD11a
TCRαβ

Fig. 4

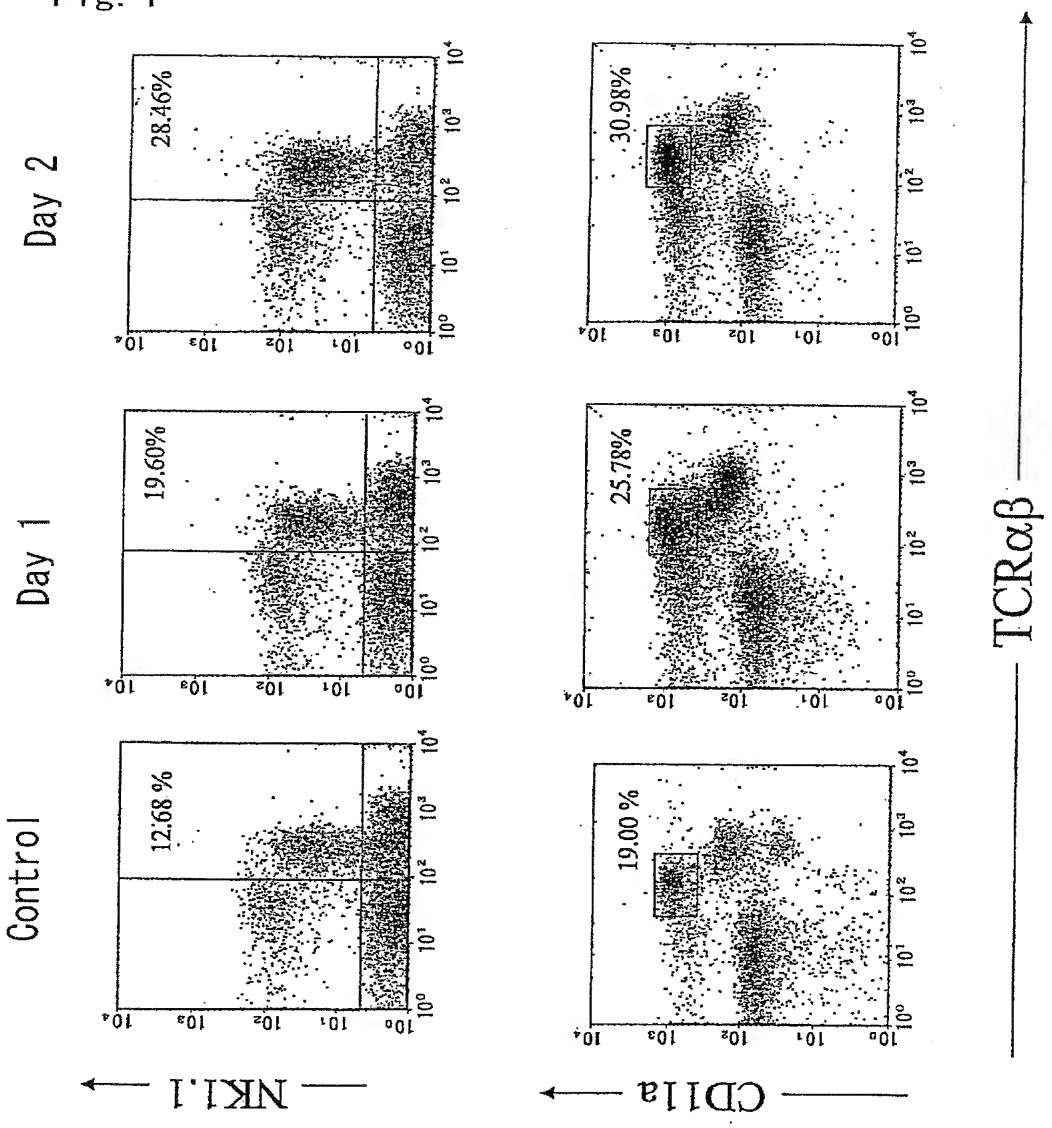

Day 2
Day 1
Control
28.46%
30.98%
19.60%
25.78%
12.68 %
19.00 %
NK1.1
CD11a
TCRαβ

Fig. 5

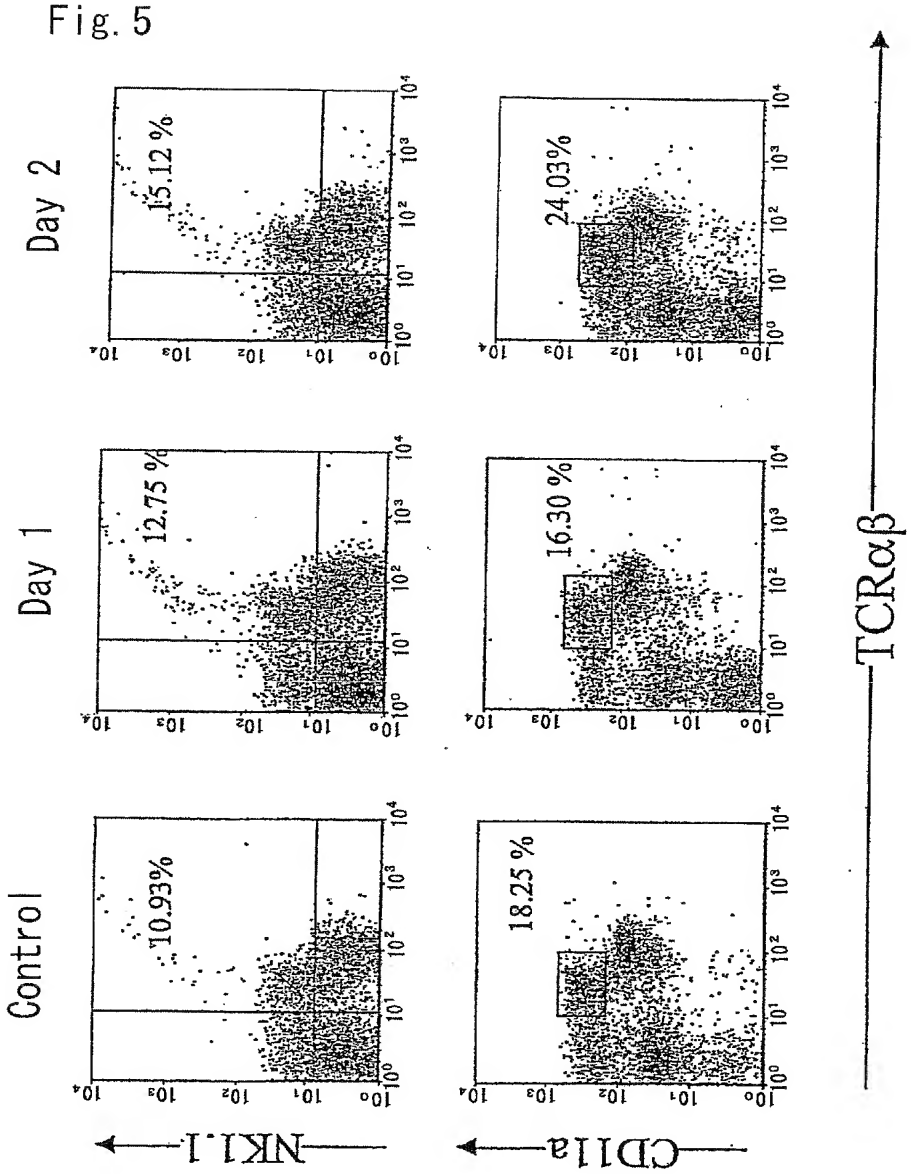

Day 2
Day 1
Control
15.12 %
24.03%
12.75 %
16.30 %
10.93%
18.25 %
NK1.1
CD11a
TCRαβ

Fig. 6

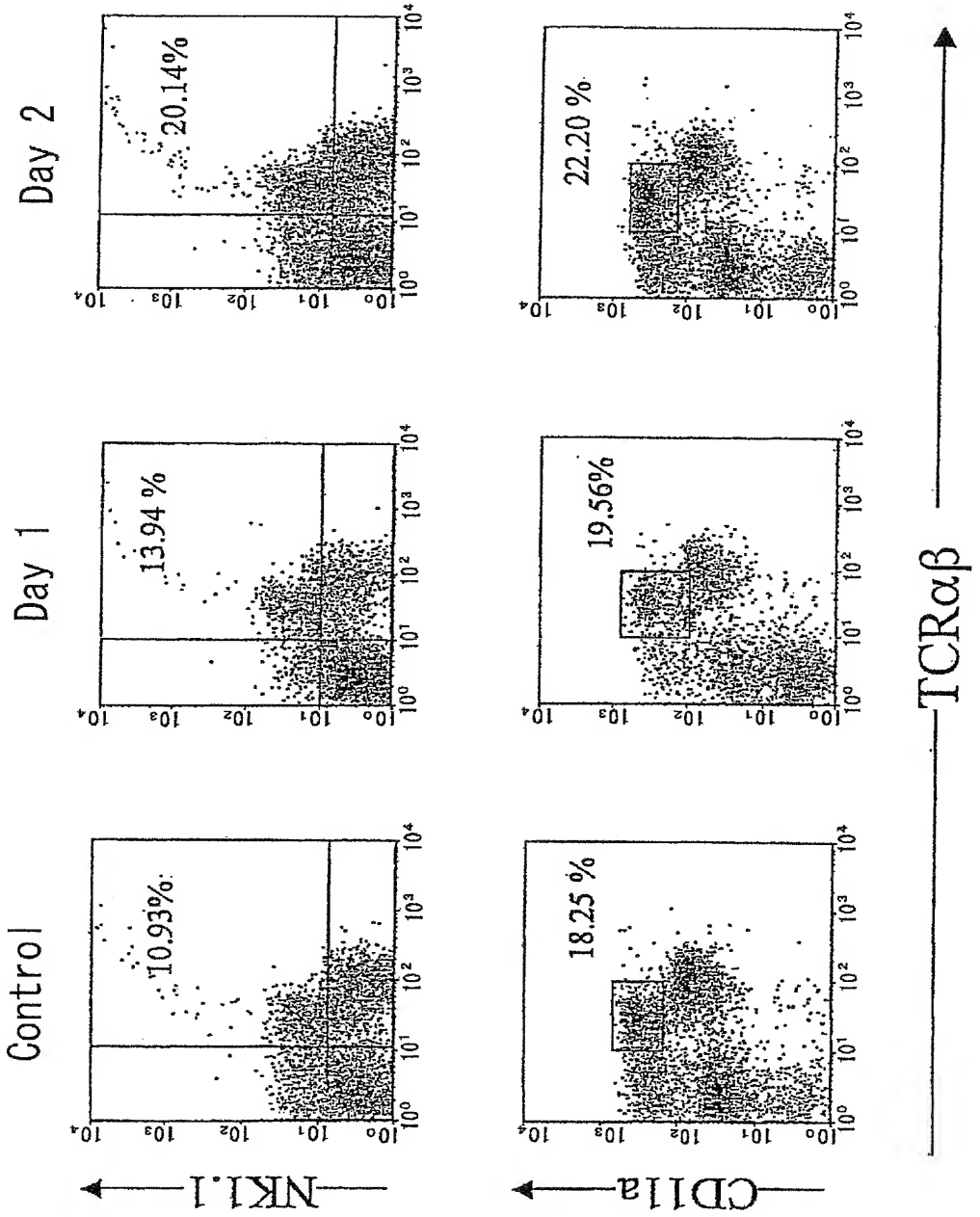

Day 2
Day 1
Control
20.14%
22.20 %
13.94 %
19.56%
10.93%
18.25 %
NK1.1
CD11a
TCRαβ

Fig. 7

	Day 1 (%)	Day 2 (%)
Control	14.5	13.7
GSL-1	18.4	22.8 *
GSL-2	21.7 *	26.1 *
GSL-6	14.6	23.4 *
GSL-7	11.9	23.2 *

Fig. 8

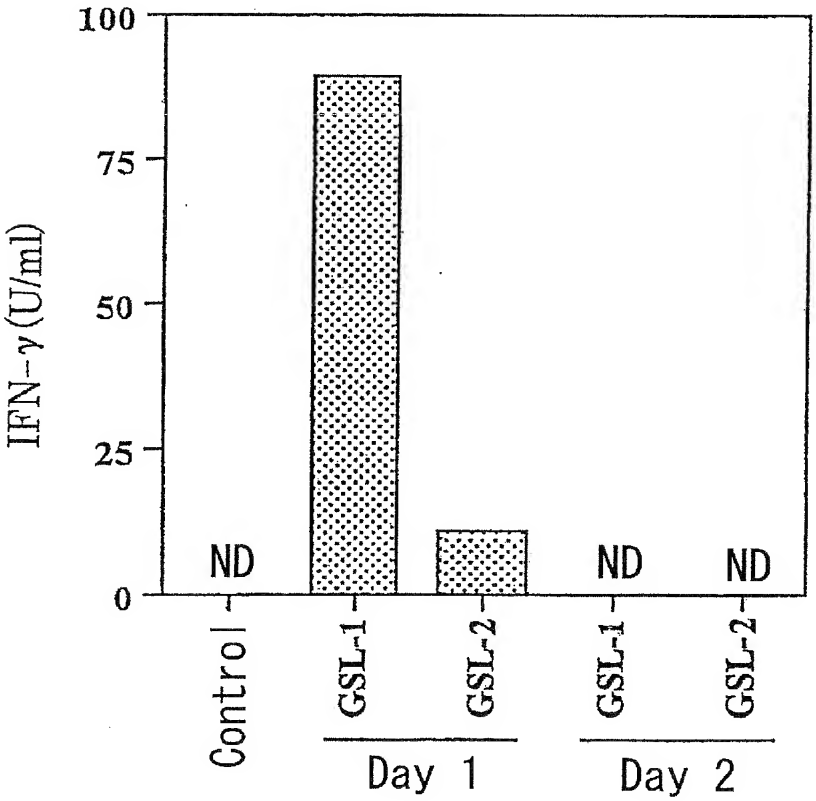
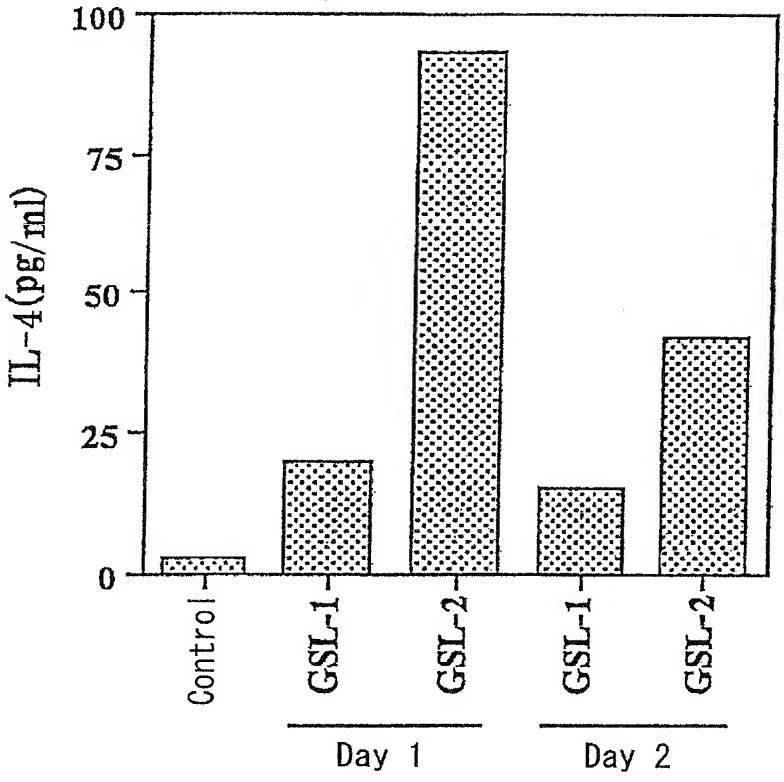


Fig. 9

	Day 1(%)	Day 2(%)
Control	3.4	3.4
GSL-1	9.8	35.5
GSL-2	24.0	25.9

Fig. 10



[Document Name] Abstract

[Abstract]

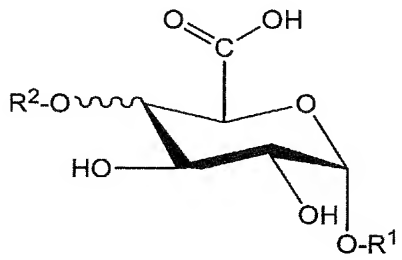
[Object] Provided is a more effective composition for NKT cell activation.

[Solving means]

A composition for NKT cell activation comprising a glycosphingolipid having a structure represented by the following formula (1):

formula (1)

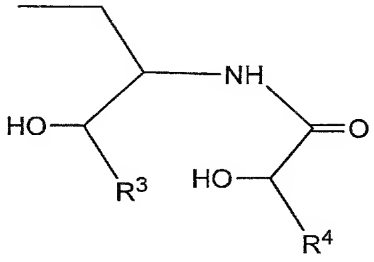
[Formula 1]



wherein R^1 represents the following formula (1-1):

formula (1-1)

[Formula 2]



wherein R^3 represents alkyl which may have cycloalkyl, or alkenyl, and R^4 represents alkyl; and

R^2 represents hydrogen, or α-galactose, α-glucose, α-mannose, α-glucosamine, β-glucosamine or a combination thereof.